L- and P-Selectin and CD11/CD18 in Intracapillary Neutrophil Sequestration in Rabbit Lungs

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Infusion of complement fragments induces rapid sequestration of neutrophils within pulmonary capillaries. This study examined the mechanisms through which this sequestration occurs, as well as the effect of complement fragments on the expression of L-selectin and CD11/CD18 using ultrastructural immunohistochemistry. Studies using anti-P-selectin antibodies, fucoidin, L-selectin-depleted neutrophils, and anti-CD18 antibodies showed that selectins and CD18 were not required for neutrophil sequestration. However, maintaining the sequestered neutrophils within the pulmonary capillaries required both L-selectin and CD11/CD18. Neutrophils in the pulmonary capillaries of rabbits given complement fragments expressed 72% less L-selectin and 98% more CD11/CD18 than did those in rabbits given saline. Shedding of L-selectin occurred preferentially from the microvillar processes of the plasma membrane rather than from the flat intervening regions. About 28% of L-selectin still remained on intracapillary neutrophil membranes after 15 min and was likely available for binding. Shedding of L-selectin appeared slower *in vivo* than *in vitro*. These studies indicate that neutrophil sequestration induced by complement fragments requires at least two sequential steps, one that does not require recognized adhesion molecules followed by a second that requires L-selectin and CD11/CD18. Kubo H, Doyle NA, Graham L, Bhagwan SD, Quinlan WM, Doerschuk CM. L- and P-selectin and CD11/CD18 in intracapillary neutrophil sequestration in rabbit lungs.

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During infection and inflammation, inflammatory mediators appear in the bloodstream. Many of these mediators bind to receptors on the surface of circulating leukocytes and endothelial cells inducing a cascade of activation pathways. These activated leukocytes, particularly neutrophils, then contribute to tissue injury by the production and release of oxidants, proteases, cytokines, and other mediators. Complement fragments, particularly C5a, are one such inflammatory mediator that bind to circulating neutrophils and cause neutropenia because of rapid sequestration of neutrophils within the microvascular beds of many organs, but particularly the lungs (1). The sequestered neutrophils are located primarily within the pulmonary capillaries, and the majority are single rather than aggregated (1, 2).

The molecular mechanisms underlying the early events in neutrophil sequestration and neutropenia remain elusive. In particular, studies examining the role of adhesion molecules have shown that the leukocyte adhesion molecule CD11/CD18 is not required for neutrophil sequestration induced by infusion of complement fragments, although it is required for these sequestered cells to remain in the lung for more than several minutes (2). Although the selectin family of adhesion molecules often mediates the initial rolling of leukocytes along the postcapillary venules in the systemic circulation (3, 4), the function of selectins in the pulmonary capillary bed is less clear. Rolling does not occur within these capillaries, presumably because their diameters are similar to or narrower than the diameters of neutrophils (5–7). However, selectin-mediated adhesion and signaling could still be important in the response of neutrophils. L-selectin plays a role in neutrophil-mediated lung injury induced by cobra venom factor, a protease that cleaves complement fragments to generate C5a (8), although recent studies from our laboratory using L-selectin-deficient mice have suggested that L-selectin is not required for complement-fragment-induced neutrophil sequestration to occur (9). P-selectin, expressed by endothelial cells as well as platelets, also plays a role in lung injury induced by cobra venom factor (10, 11). E-selectin is unlikely to play a role in the rapid onset of neutrophil sequestration because it is not constitutively expressed by endothelial cells, and its upregulation requires protein synthesis.

The binding of inflammatory stimuli to neutrophil receptors alters the expression and activation state of L-selectin and CD11/CD18 on the plasma membrane (12). In quiescent neutrophils, the density of L-selectin is greater on the plasma membrane of the microvillar processes than it is on the intervening flat regions (13–15). After stimulation by mediators,

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L-selectin is proteolytically shed from the surface (12, 16–18). *In vitro*, shedding is nearly complete by 1 to 5 min of stimulation by C5a, fMLP, PMA, or other mediators (12, 16). *In vivo*, shedding also occurs and can be complete either before emigration or after transmigration has occurred (14). Whereas CD11a/CD18 is expressed only on the plasma membrane, CD11b/CD18 is expressed both on the plasma membrane and on several populations of granules, and its expression can be upregulated during activation (12–15).

These studies tested the hypothesis that selectins are important in neutrophil sequestration induced by complement fragments using two approaches. First, isolated, radiolabeled rabbit neutrophils were treated with chymotrypsin under conditions known to induce shedding of L-selectin and not other adhesion molecules (19), and the ability of these L-selectin-depleted neutrophils to sequester was evaluated. Second, the effect of pretreatment with either a blocking anti-P-selectin antibody, fucoidin (an inhibitor of selectins) (20, 21), or fucoidin combined with an anti-CD18 antibody on complement-induced neutrophil sequestration was also determined. These studies also quantitated complement-fragment-induced changes in the expression of L-selectin and CD11/CD18 on neutrophils sequestered within the pulmonary capillaries and determined the site of L-selectin shedding on the neutrophil plasma membrane using ultrastructural immunohistochemistry with colloidal gold labeling.

METHODS

Preparation of Complement-activated Plasma

Zymosan-activated plasma was used as a source of complement fragments and was prepared as previously described (1, 2). In brief, zymosan A yeast (Z-4250; Sigma Chemical, St. Louis, MO) was boiled for 15 min and centrifuged twice. Heparinized blood obtained from donor rabbits was centrifuged at 1,200 × *g*. The plasma was incubated with the boiled zymosan yeast (5 mg/ml plasma) for 30 min at 37° C. The activated plasma was centrifuged twice at 1,200 × *g* for 10 min to remove the yeast and was used within 1 h of preparation.

Protocol 1: Sequestration of Chymotrypsin-treated Neutrophils

Neutrophil isolation and radiolabeling. Blood was drawn from the central ear artery of donor rabbits, and the neutrophils were isolated and radiolabeled as previously described (1). In brief, the blood was anticoagulated with acid-citrate-dextrose, and the red blood cells were sedimented using dextran (100 to 200 kD, 4%) at a final concentration of 1.9%. After the residual red blood cells were hypotonically lysed, the neutrophils were separated from the mononuclear cells by centrifugation through Histopaque (Sigma, 1077-1). The isolated neutrophils were radiolabeled with ^{111}Indium oxine (50 μCi/2 × 10^7 neutrophils) for 10 min at room temperature. After washing with buffer twice, the neutrophils were resuspended at a concentration of 2 to 5 × 10^6 cells/ml. The neutrophils were more than 95% pure. Aliquots of the ^{111}In-neutrophils were sampled to determine the total number of ^{111}indium cpm injected into each rabbit.

Chymotrypsin digestion. A stock solution of chymotrypsin (Sigma, C-4129) was prepared at a concentration of 10 U/ml Krebs buffer containing calcium and magnesium. Half the neutrophils in each isolate were treated with chymotrypsin at a final concentration of 1 U/10^6 neutrophils for 10 min at 37° C (19). The other half was similarly incubated with buffer. Samples (100 μl) were taken and cytospins were prepared to determine the expression of L-selectin using immunocytochemistry (*see below*). The neutrophils were immediately injected into prepared rabbits.

Protocol. Female New Zealand white rabbits weighing 2.4 to 2.7 kg (n = 110) were anesthetized intramuscularly with ketamine hydrochloride (70 to 100 mg/kg) and acepromazine maleate (8 to 10 mg/kg) with additional injections as needed to maintain anesthesia. A catheter was placed in the proximal aorta through the carotid artery. A butterfly catheter was placed in a marginal ear vein.

For each neutrophil isolation (n = 5 total), two animals were studied, one of which received ^{111}In-neutrophils that had been treated with chymotrypsin for 10 min and the other received buffer-treated ^{111}In-neutrophils. Blood was sampled before and 2, 5, 10, and 15 min after the ^{111}In-neutrophils were injected. After they had circulated for 15 min, an infusion of complement fragments was begun through the ear vein catheter at a rate of 0.3 ml/min/kg for 15 min. Blood samples were obtained before and at 1, 2, and 4 min after the infusion was begun to measure the levels of ^{111}indium counts bound to neutrophils.

The fraction of neutrophil-bound ^{111}indium was determined by measuring radioisotope levels in whole blood and plasma samples from each time point. The ^{111}In-neutrophil cpm were then calculated as:

$$\frac{^{111}\text{In-neutrophil cpm}}{\text{g blood}} = \frac{^{111}\text{In cpm}}{\text{g whole blood}} - \frac{^{111}\text{In cpm}}{\text{g plasma}} \times (1\text{-hematocrit}). \quad (1)$$

The neutrophil-bound ^{111}indium counts were normalized for comparison between animals by dividing the ^{111}In-neutrophil cpm/g blood by the total number of ^{111}indium counts injected into each rabbit. The normalized counts were expressed as a percent of the baseline value immediately before the infusion of complement fragments was begun to allow comparison with the change in unlabeled native circulating neutrophil counts.

Immunocytochemistry. Immunocytochemical techniques were used to determine if chymotrypsin cleaved L-selectin from the isolated, radiolabeled neutrophils. After the cytospins were warmed to room temperature, they were fixed with acetone:methanol 1:1 for 90 s and transferred to 0.05 M TRIS-buffered saline (pH, 7.6). The alkaline phosphatase-antialkaline phosphatase technique was used to localize the primary antibodies (22). In brief, the sections were incubated with either the murine monoclonal antihuman L-selectin antibody DREG 200, which crossreacts with rabbit L-selectin (kindly provided by Dr. Eugene C. Butcher), the murine monoclonal antihuman CD18 antibody, 60.3, that crossreacts with rabbit CD18 (Bristol Myer Squibb, Seattle, WA), or nonspecific mouse immunoglobulin G for 30 min. After washing with TRIS-buffered saline, antimouse immunoglobulin (Dakopatts Z259; Dako Corp., Carpinteria, CA) was applied for 30 min, followed by the alkaline phosphatase-antialkaline phosphatase complex (D651; Dako) 30 min. After repeating the incubations with the secondary antibody and the complex, the alkaline phosphatase substrate containing new fuchsin was added for 20 min. After washing, the slides were counterstained with hematoxylin and mounted in aqueous mounting medium. The cytospins prepared from chymotrypsin and buffer-treated ^{111}In-neutrophils were compared to determine if chymotrypsin had produced cleavage of L-selectin.

Protocol 2: The Effect of Anti-P-Selectin Antibody, Fucoidin, and Fucoidin Combined with Anti-CD18 Antibody on Neutrophil Sequestration

Anti-P-selectin antibody (PB1.3) was kindly provided by R. M. Rose, Cytel Corp. This antibody was generated against human P-selectin and cross-reacts with canine, rat, and rabbit P-selectin (5, 10). Fucoidin inhibits P-selectin and L-selectin (20, 21). The anti-CD18 antibody was 60.3 (2, 23).

New Zealand white rabbits were anesthetized and prepared as described in Protocol 1. Either anti-P-selectin antibody (2 mg/kg, n = 5), fucoidin (5 mg/kg, n = 5), fucoidin and anti-CD18 antibody (2 mg/kg, n = 3), or mouse IgG (2 mg/kg, n = 5) was injected intravenously. After 15 min, an infusion of complement fragments or plasma (0.3 ml/kg/min) was begun through the ear vein. A blood sample was taken from the arterial line prior to injection of antibody or fucoidin, 1 min before infusion of complement fragments or plasma, and at 1, 2, 4, 7, 10, and 15 min during the infusion to determine the number of circulating neutrophils. At 15 min, saturated potassium chloride was injected into the carotid line to stop the heart, the chest was rapidly opened, the base of the heart was tied to keep the pulmonary blood volume in the lungs, and the lungs were fixed by intratracheal instillation of 6% glutaraldehyde in phosphate buffer at 25 cm H_2O pressure. After 1 h, the lungs were removed and allowed to fix overnight.

Lung tissue blocks were prepared from the midportion of the left lower lobe, and methacrylate-embedded sections measuring 1 to 2 μm in thickness were cut and stained with hematoxylin-eosin. The sections were examined at oil magnification ×600 and the number of intracapillary RBC and neutrophils were counted in five randomly selected fields of peripheral lung tissue. The number of intracapillary neutrophils was expressed per 1,000 RBC (2).

Protocol 3: Ultrastructural Localization of L-Selectin and CD11/CD18

Infusion of complement fragments. Anesthetized rabbits weighing 2.4 to 2.7 kg (n = 10) prepared as described in Protocol 1 received infusions of complement fragments (0.3 ml activated plasma/min/kg, n = 5) or saline (n = 5) for 15 min. Blood samples for circulating leukocyte counts and differentials were taken before and at 14.5 min. At 15 min, the heart was stopped by intra-arterial injection of saturated potassium chloride.

The animal's chest was rapidly opened, the base of the heart was tied, and 0.025% glutaraldehyde in phosphate-buffered saline (PBS) was instilled through the trachea at 30 cm H_2O pressure (14). The lungs were removed and allowed to fix for 1 h at 4° C. The lung tissue was then sectioned into 1 × 1 × 1 mm cubes and fixed for an additional 60 min. After rinsing in PBS, the tissue sections were infiltrated with 2.5 M sucrose overnight at 4° C. Each tissue fragment was then placed on a aluminum specimen pin and frozen in liquid nitrogen.

Cryoultramicrotomy and immunogold labeling. Sections measuring 90 to 110 nm were cut using a cryoultramicrotome and collected on formvar-coated copper grids. The grids were inverted on 40-μl droplets of PBS containing 5% fetal calf serum (PBS-FCS).

Either L-selectin or CD18 was localized using immunogold labeling techniques as previously described (14). The cryosections were incubated with either the anti-L-selectin (DREG200) or the anti-CD18 antibody (60.3) at a concentration of 0.01 mg/ml in PBS-FCS. To assess nonspecific gold labeling, additional cryosections were incubated with nonimmune mouse IgG (Sigma) at the same concentration. After washing in PBS-FCS, the sections were incubated with a colloidal gold-labeled antimurine IgG antibody (1:26 dilution; Sigma). After postfixing in 1% glutaraldehyde, the sections were embedded and contrasted with 1.8% methylcellulose containing 0.3% uranyl acetate in distilled water.

Immunogold quantitation. The immunogold-labeled tissue sections were examined using a Phillips CM 10 transmission electron microscope. Neutrophils were identified by their segmented nuclear lobes and cytoplasmic granules. All intracapillary neutrophils were photographed at magnification ×27,000 to 32,000. In each animal (n = 5 in each group) at least five neutrophils were examined.

The density of gold particles was quantified by counting the number of gold particles along free plasma membrane lengths using the photographic negatives (14). The length of plasma membrane was measured on prints using a digitizing tablet connected to a personal computer running SigmaScan software (Jandel Scientific, Corte Madera, CA). The digitizing tablet was calibrated prior to each use using a similarly enlarged photographic image of an electron microscope diffraction grating. The gold particle density of each neutrophil profile was calculated by dividing the number of gold particles by the length of plasma membrane examined (14). An average value of gold particles/μm neutrophil plasma membrane was determined for each animal. The reported values are the mean and standard deviation for the groups based on the average value for each animal.

Gold particles were categorized as on the microvillar processes or on the intervening flat regions of membrane using cryosections of neutrophils labeled with either anti-L-selectin or anti-CD18 antibody. For each profile, the number of gold particles on microvillar processes or on the intervening flat regions of membrane was counted. The ratio of gold particles on microvillar processes/flat region was calculated for each profile. The average ratio of microvillar process/flat region was calculated for each animal, and the mean and standard deviation for the complement fragment and saline-treated groups were compared.

Protocol 4: Localization of L-Selectin Using Light Immunohistochemistry

To determine if L-selectin cleavage differed *in vivo* and *in vitro*, L-selectin expression was evaluated semiquantitatively and compared on isolated rabbit leukocytes with or without incubation with complement fragments and on intracapillary neutrophils in lungs from rabbits given infusion of complement fragments or saline *in vivo*.

Preparation of rabbit leukocytes. Leukocytes from five rabbits were prepared by incubating rabbit blood anticoagulated using acid-citrate-dextrose with dextran (100 to 200 kD, 4%) at a final concentration of 1.9% to sediment the RBC as previously described. The supernatant was centrifuged and the residual RBC were hypotonically lysed. The leukocytes were suspended in PBS at a concentration of 2 × 10^6/ml. Samples were incubated for 14 min with either complement fragments (final concentrations either 0.3 or 3% activated plasma) or saline at 37° C. Cytospins were immediately prepared and stored at −80° C. Prior to staining, they were warmed to room temperature and fixed using acetone:methanol 1:1 for 90 s and transferred to 0.05 M TRIS-buffered saline (pH, 7.6).

Preparation of lung tissue. Rabbits received an infusion of saline (n = 5) or complement fragments (n = 5) as described above except that after the lungs were removed, they were inflated with tissue tek (OCT compound; Miles Laboratory, Elkhart, IN) mixed with saline 1:1 prior to freezing in liquid nitrogen. Frozen sections measuring 7 to 8 μm were cut using a cryostat, collected on aminopropyltriethoxy-saline-treated glass slides, fixed in acetone for 10 min, and transferred to 0.05 M TRIS-buffered saline (pH, 7.6).

Light immunohistochemistry. L-selectin was localized using several concentrations of the anti-L-selectin antibody, DREG200. The alkaline phosphatase-antialkaline phosphatase technique was used to localize the primary antibodies (22). In brief, the cytospins and histologic sections were incubated with DREG200 (20,000, 6,700, 2,000, 670, 200, 100, 50, or 25 ng/ml) or nonspecific mouse immunoglobulin G (20,000 ng/ml) for 30 min. Immunostaining and counterstaining were performed as described in Protocol 1.

The lung sections and cytospins were examined to determine the dilution at which the L-selectin staining on neutrophils was lost in the complement fragment and the saline-treated groups. The data are shown as the mean dilution ± SEM at which staining was no longer visible.

TABLE 1

NUMBERS OF CIRCULATING ^{111}In-NEUTROPHILS AFTER INJECTION PRIOR TO INFUSION OF COMPLEMENT FRAGMENTS OR PLASMA*

Time after Injection (min)	Normalized ^{111}In-neutrophil cpm†		Percent of Baseline‡	
	Chymotrypsin-treated	Buffer-treated	Chymotrypsin-treated	Buffer-treated
2	280 ± 118	305 ± 121	186 ± 48%	187 ± 15%
5	220 ± 87	215 ± 87	130 ± 21%	126 ± 3%
10	235 ± 68	180 ± 86	101 ± 6%	97 ± 7%
15	186 ± 58	179 ± 78	100%	100%

* Chymotrypsin-treated (1 U/10^6 neutrophils for 10 min at 37° C) and buffer-treated ^{111}In-neutrophil circulated similarly in the first 15 min after injection. Data are expressed as mean ± SEM (n = 5 rabbits in each group).

† The ^{111}In-neutrophil cpm were normalized for the total number of ^{111}In-neutrophil cpms injected and are expressed × 10^6/ml blood.

‡ The circulating ^{111}In-neutrophil cpm are expressed as a percentage of the circulating cpm at 15 min, immediately prior to the infusion of complement fragments or plasma.

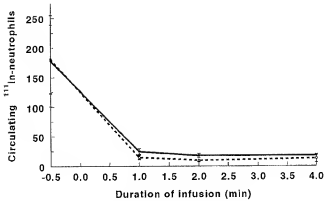

Figure 1. The effect of infusion of complement fragments on circulating radiolabeled neutrophil counts after treatment with chymotrypsin (*closed circles, solid line*) or buffer (*open circles, dotted line*). Cleavage of L-selectin using chymotrypsin did not prevent the decrease in circulating neutrophil counts induced by infusion of complement fragments. The values are expressed as normalized by the counts injected $\times 10^{-6}$/ml, as described in METHODS.

Statistics

Repeated analysis of variance was used to compare the leukocyte counts over time and the L-selectin and CD11/CD18 expression and distribution. The modified Bonferroni correction was used to correct for multiple comparisons when significant overall differences were identified. A nonparametric ranking test (Mann-Whitney) was used to compare the dilutions of antibody at which L-selectin staining disappeared. A probability of less than 0.05 for the null hypothesis was accepted as indicating a statistically significant difference. Data are expressed as mean ± SEM unless otherwise noted.

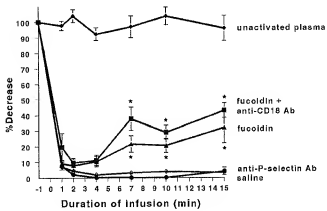

Figure 2. Circulating neutrophil counts after infusion of complement fragments or unactivated plasma. All animals except the group labeled "unactivated plasma" received infusion of complement fragments immediately after the initial blood sampling. Intravascular complement fragments induced a decrease in circulating neutrophil counts that was similar in rapidity and degree when animals were pretreated with anti-P-selectin antibody, fucoidin, fucoidin combined with anti-CD18 antibody, or saline. However, by 4 min of infusion, the circulating neutrophil count began to increase when the rabbits were pretreated with either fucoidin or fucoidin combined with anti-CD18 antibody. These two pretreatments were not significantly different. *Asterisks* indicate values significantly greater than saline pretreated rabbits and less than rabbits given infusion of unactivated plasma, $p < 0.05$.

RESULTS

Protocol 1: Sequestration of Chymotrypsin-treated Neutrophils

The ^{111}In-neutrophils that were treated with chymotrypsin showed no staining for L-selectin using immunocytochemical techniques. In contrast, the buffer-treated ^{111}In-neutrophils showed bright staining. Staining for CD18 showed similar intensities in both groups. Similar numbers of chymotrypsin-treated and buffer-treated ^{111}In-neutrophils were circulating during the first 15 min after injection (Table 1). The circulating ^{111}In-neutrophil counts before and at 1, 2, and 4 min of infusion of complement fragments or plasma are shown in Figure 1. Both the chymotrypsin-treated and the buffer-treated ^{111}In-neutrophil counts decreased at a similar rate and degree during the infusion of complement fragments.

Protocol 2: The Effect of Anti-P-selectin Antibody, Fucoidin, and Fucoidin Combined with Anti-CD18 Antibody on Neutrophil Sequestration

Infusion of complement fragments induced a rapid and profound fall in the number of circulating neutrophils by 1 min, and this neutropenia continued for the duration of the 15-min infusion (Figure 2). Neither anti-P-selectin antibody nor fucoidin nor fucoidin combined with anti-CD18 antibody had any effect on either the degree or the rate of this decrease in circulating neutrophil counts (Figure 2). However, the circulating neutrophil counts began to increase in the blood of rabbits pretreated with either fucoidin or fucoidin and anti-CD18 antibody by 4 min despite continuous infusion of complement fragments (Figure 2) and recovered to 30 to 40% of the initial counts by 15 min. Treatment with combined fucoidin and anti-CD18 antibody tended to result in a faster recovery, although this trend was not statistically significant. Anti-P-selectin antibody had no effect on either the initial or the later sequestration.

When the lungs of these animals were evaluated after 15 min of infusion, the number of accumulated neutrophils in-

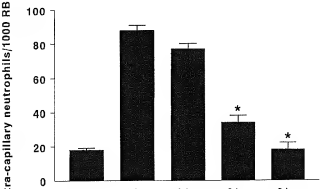

Figure 3. Neutrophil sequestration in the lungs after a 15-min infusion of complement fragments or unactivated plasma. Complement fragments induced a 4- to 5-fold increase in the number of neutrophils sequestered within the pulmonary capillaries ($p < 0.05$). Pretreatment with anti-P-selectin antibody had no effect. Pretreatment with either fucoidin or fucoidin combined with anti-CD18 antibodies inhibited this sequestration. C frag = complement fragments. *Asterisks* indicate values significantly less than rabbits given infusion of complement fragments and no pretreatment, $p < 0.05$.

TABLE 2
CIRCULATING NEUTROPHIL COUNTS ($\times 10^6$/ml BLOOD)

	Before Infusion	After 14.5 min
Saline-treated	1.5 ± 0.3	1.4 ± 0.3
Complement fragment–treated	1.6 ± 0.4	0.2 ± 0.1*

* Significantly different from counts before infusion, $p < 0.05$.

creased dramatically by more than 5-fold in the control rabbits that received complement fragments (Figure 3). Anti-P-selectin antibody had no effect on this increase. However, fucoidin inhibited this accumulation by 78%, and pretreatment with both fucoidin and anti-CD18 antibody completely prevented this accumulation (Figure 3).

Protocol 3: Ultrastructural Localization of L-Selectin and CD11/CD18

The number of circulating neutrophils before and during infusion of saline or complement fragments is shown in Table 2.

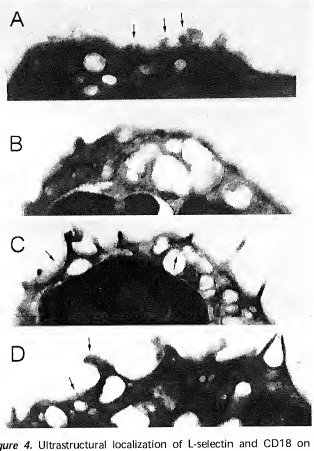

Figure 4. Ultrastructural localization of L-selectin and CD18 on neutrophils within the pulmonary capillaries of rabbits treated intravascularly with infusions of complement fragments or saline for 15 min. L-selectin expression was located primarily on the microvillar processes of neutrophils in the lungs of rabbits given saline (*A*). Complement fragments caused shedding of L-selectin that occurred preferentially from the microvillar processes rather than the flat intervening regions (*B*). CD18 was present on the membrane and in granules of neutrophils from saline-treated rabbits (*C*). Complement fragments caused upregulation of CD18 on both the microvillar processes and the flat regions (*D*). Gold particles: 10 nm.

As previously demonstrated, infusion of complement fragments induced a significant reduction in the number of circulating neutrophils at 14.5 min.

The distribution of L-selectin and CD18 on intracapillary neutrophils using ultrastructural immunohistochemistry is shown in Figures 4 and 5. Infusion of complement fragments resulted in a significant decrease in the expression of L-selectin on intracapillary neutrophils compared with that found on neutrophils in the lungs of rabbits given infusion of saline (Figure 5). However, even after a 15-min infusion of fragments, 28% of the L-selectin was still present. The L-selectin on neutrophils from the lungs of control rabbits was preferentially located on the microvillar processes compared with the flat regions. The ratio of the number of gold particles found on microvillar processes to the number on flat regions was 3.7 (Figure 6). Infusion of complement fragments resulted in a decrease in this ratio to 1.4, indicating that a greater percentage of the L-selectin was shed from microvillar processes than from the flat regions (Figure 6).

The amount of CD18 expression increased by 96% after infusion of complement fragments (Figure 5). In contrast to L-selectin, CD18 was not preferentially localized to the microvillar process but was present on both the processes and the flat regions. In addition, complement fragments had no effect on the ratio of gold particles on microvillar processes to that on the flat regions (ratio of neutrophils from saline-treated rabbits, 0.67 ± 0.13; from complement-fragment-treated rabbits, 0.68 ± 0.09) (Figure 6). As previously described, CD18 was also present within the membranes of at least one population of granules (13–15).

Sections of lung tissue stained using nonimmune murine IgG showed virtually no background staining. The number of gold particles was less than 0.1 particles/μm neutrophil membrane, and many neutrophils had no gold particles bound. When the anti-L-selectin or anti-CD18 antibodies were used, virtually all gold particles were associated with the plasma membrane, and no nonspecific adhesion to the tissue sections was observed.

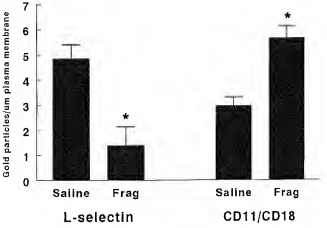

Figure 5. Expression of L-selectin and CD18 on the plasma membrane of intracapillary neutrophils within the pulmonary capillaries of rabbits treated with complement fragments or saline. Complement fragments induced shedding of L-selectin (72%) and upregulation of CD18 (96%). *Asterisks* indicate significant difference from expression on neutrophils from saline-treated rabbits.

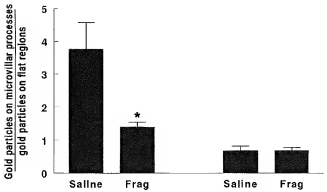

Figure 6. Expression of L-selectin and CD18 on the microvillar processes compared with the flat regions of the membrane on intracapillary neutrophils within the pulmonary capillaries of rabbits treated with complement fragments or saline. The ratio of L-selectin expression on microvillar processes to flat regions decreased after infusion of complement, indicating that L-selectin was preferentially cleaved from the processes. In contrast, complement fragments did not change the ratio of CD18 expression at these sites. *Asterisk* indicates significant difference from expression on neutrophils from saline-treated rabbits.

Protocol 4: Localization of L-Selectin Using Light Immunohistochemistry

Light immunohistochemical studies were performed to compare L-selectin expression on intracapillary neutrophils in lung tissue from rabbits given complement fragments or saline *in vivo* with that on isolated neutrophils exposed to complement fragments or saline *in vitro* by determining the concentration of anti-L-selectin antibody at which staining disappeared. These studies showed that staining of L-selectin on neutrophils in the pulmonary capillaries disappeared at similar concentrations of anti-L-selectin antibody in the lungs of saline-treated and complement fragment–treated rabbits ($p > 0.05$) (Table 3). However, ultrastructural studies discussed above demonstrated a 72% decrease in L-selectin expression using the same antibody. These data suggest that the APAAP technique, which is designed to be a very sensitive but not quantitative technique, cannot detect as great as a 72% decrease in expression.

In contrast to the light immunohistochemical studies of lung section, the staining of L-selectin on isolated neutrophils treated with either low (0.3%) or high (3.0%) concentrations of complement fragments *in vitro* was lost at a higher concentration of anti-L-selectin antibody than on neutrophils treated with saline (Table 3). At low concentrations of complement fragments (0.3%), L-selectin staining was lost at lower concentrations of antibody than when higher concentrations of complement fragments (3.0%) were used (Table 3).

TABLE 3

CONCENTRATION OF ANTI-L-SELECTIN ANTIBODY AT WHICH STAINING OF NEUTROPHILS WAS LOST*

	Concentration (*ng/ml*)
Neutrophils within pulmonary capillaries from rabbits treated *in vivo*	
Saline	30 ± 5
Complement fragments	34 ± 7
Neutrophils treated *in vitro*	
Saline	65 ± 15
Complement fragments, 0.3%	667†
Complement fragments, 3%	3,240 ± 1,430†‡

* Values are means ± SEM.
† $p < 0.05$ compared with neutrophils treated with saline.
‡ $p < 0.05$ compared with neutrophils treated with 0.3% complement fragments.

DISCUSSION

This study investigated the response of neutrophils to intravascular complement fragments. Using several approaches, the results show that neither L-selectin nor P-selectin nor CD11/CD18, either singly or in combination, are required for the initial rapid sequestration and neutropenia that occur soon after neutrophils are activated. In contrast, adhesion molecules are required to maintain the sequestered neutrophils within the lungs. CD11/CD18 is clearly required, as inhibiting this molecule alone prevented prolonged sequestration of neutrophils in the lung (2). The data presented in this report show that either L-selectin itself or L-selectin in combination with P-selectin is also required for sequestered neutrophils to remain in the pulmonary capillaries, as evaluated using fucoidin. P-selectin alone cannot mediate either process, as demonstrated by the lack of an effect of the anti-P-selectin antibody. Because E-selectin is not found on unstimulated endothelial cells and requires protein synthesis for expression, it is very unlikely to mediate sequestration, which occurs within 1 min. Taken together, these results suggest that presently recognized adhesion molecules are not required for neutrophils that are activated in the bloodstream to sequester in the lung.

This study suggests that neutrophil sequestration occurs through at least two sequential steps, each requiring a distinct mechanism. First, neutrophils sequester through a process that does not require recognized adhesion molecules. Second, after 4 to 7 min, L-selectin and CD11/CD18 engage their endothelial cell ligands and are required to keep the neutrophils sequestered within the pulmonary capillaries. CD11/CD18 is presumably binding to constitutively expressed ICAM-1. The ligand for L-selectin is unclear. It is unlikely to be P-selectin (13), as anti-P-selectin antibodies did not inhibit sequestration and P-selectin does not appear to be constitutively expressed by capillary endothelium (10, 11). To our knowledge, no ligand for neutrophil L-selectin has yet been demonstrated on flat endothelium.

The mechanism through which the immediate sequestration occurs remains elusive. Changes in biomechanical properties that result in stiffening of neutrophils and a decrease in their ability to deform is the most likely mechanism. Many investigators have shown that neutrophils are less deformable after stimulation by a number of mediators including IL-8, fMLP, and complement fragments (24–26). This increase has been attributed to a stimulus-induced increase in the polymerization of g-actin to form f-actin in the submembrane region of neutrophils. Recent data suggest that the changes in actin concentration within regions of neutrophils also occur, causing a redistribution of actin from the central region to the submembrane region and the microvillar processes (27). In traveling through the capillary pathway from pulmonary arteriole to venule, almost all neutrophils will encounter a capillary segment that is narrower than the spherical diameter of neutrophils. Therefore, most neutrophils must deform at least a small amount to pass through the pulmonary capillary bed (5–7). If neutrophils become stiffer and lose their ability to deform, they will be trapped within the capillary bed. This hypothesis is supported by recent data showing that a larger fraction of

the intracapillary neutrophils are round after infusion of complement fragments for 1.5 min (27). This increase in spherical neutrophils is similar to the number of circulating neutrophils that sequestered within 1.5 min (27).

Changes in the volume of neutrophils induced by inflammatory mediators, including C5a and fMLP, occur and are thought to be mediated by uptake of sodium and water through the Na^+/H^+ antiport (28–30). However, the time required for these changes to occur is 4 to 30 min, considerably longer than the 30 s required for sequestration to occur. In addition, the increase in volume has been estimated to be 20 to 40%, which results in a change in diameter of only 6 to 12%, which is unlikely to result in the virtually complete neutropenia that is observed immediately after infusion of complement fragments. For these reasons, it is unlikely that volume changes play a significant role in the initial sequestration of neutrophils. Similarly, changes in shape also require longer periods of time. Recent studies have shown that neutrophils in the pulmonary capillaries are actually more spherical after 1.5 min of infusion of complement fragments (27), and no light microscopic or ultrastructural evidence of pseudopodia or other projections was observed.

After neutrophils are sequestered within the pulmonary capillaries, our study shows that interactions between adhesion molecules on neutrophils and endothelial cells occur and are mediated through L-selectin and CD11/CD18. In the systemic circulation, members of the selectin family of adhesion molecules are thought to mediate the initial rolling of neutrophils along the postcapillary venules. However, rolling does not occur in the pulmonary capillary bed (5). The role of L-selectin may instead be to mediate interactions of another nature, most likely to induce signaling and further activation (31). Despite partial shedding of L-selectin, about 28% of the L-selectin still remains on the membrane available for interactions with the endothelial cells at 15 min of infusion, suggesting that sufficient L-selectin is likely to be present at 4 to 7 min after neutrophil activation to mediate L-selectin-dependent events.

The shedding of L-selectin occurs through proteolysis between Lys321 and Ser322 in a region linking the second short consensus repeat with the transmembrane domain (16–18). The required protease appears to be a metalloprotease present on the neutrophil membrane (32, 33). Interestingly, comparison of the ratio of gold particles on microvillar processes to those on flat regions of the membrane showed that this ratio was less on neutrophils after activation by complement fragments, suggesting that L-selectin was preferentially lost from the microvillar processes during activation by complement fragments. The increased susceptibility to proteolysis of the L-selectin located on microvillar processes may be due to preferential localization of the protease on these processes. Alternatively, the L-selectin located on the microvillar processes may be more susceptible to cleavage than L-selectin located on the flat regions. Finally, the higher density of L-selectin could facilitate the crosslinking of L-selectin, which Palecanda and colleagues (34) have shown enhances its shedding. Although our data suggest that the susceptibility of L-selectin to proteolysis is dependent on the location of L-selectin within the plasma membrane, the mechanisms involved are not yet clear.

In parallel with the shedding of L-selectin, the expression of CD18 increased by approximately twofold. This upregulation is presumably due to the fusion of granular membranes containing high densities of CD11b/CD18 to the plasma membrane (14, 15, 35). The observation that the ratio of CD18 expression on microvillar processes to flat regions is similar on neutrophils from complement fragment and saline-treated animals suggests that the upregulated CD11/CD18 rapidly distributes within the membrane rather than remaining localized at the site of granule fusion. These data extend those of Erlandsen and colleagues (35) who showed that, *in vitro*, upregulated expression of CD11/CD18 occurred on both the flat regions and the microvillar processes using high resolution scanning electron microscopy.

The immunohistochemical studies at the level of light microscopy demonstrate that L-selectin staining on neutrophils was lost at a higher concentration of anti-L-selectin antibody when leukocytes were exposed to complement fragments *in vitro* compared with *in vivo* (Table 3). These studies suggest that L-selectin may be shed less easily *in vivo* than *in vitro*. Perhaps the protease that cleaves L-selectin is partially inhibited by antiproteases in the blood. One concern is that the concentration of complement fragments in the pulmonary capillary blood in the *in vivo* studies is difficult to calculate and to compare with the *in vitro* studies. An estimate of this concentration that takes into account the rate of infusion and the clearance of complement fragments from the circulation with a half-life of 4 min indicates that at 15 min after infusion, the concentration of complement fragments in the blood is 2.6% and that the concentration is greater than 0.5% by 2 min of infusion. This estimate compares with the 0.3 and 3% concentrations of complement fragments used in the *in vitro* studies. Isolated neutrophils exposed to either of these concentrations showed a significant increase in the concentration of antibody required for staining (Table 3), suggesting that shedding of L-selectin did occur *in vitro* at concentrations comparable or less than those occurring *in vivo*. For these reasons, the differences between the *in vivo* and the *in vitro* exposures to complement fragments suggest that L-selectin is cleaved more slowly *in vivo*.

In summary, these studies show that neutrophil sequestration induced by infusion of complement fragments requires at least two sequential steps. An immediate sequestration of neutrophils occurs within 1 min and does not require known adhesion molecules, most likely resulting from biomechanical changes that decrease the ability of neutrophils to deform and pass through narrow capillary segments. L-selectin and CD11/CD18-mediated interactions between neutrophils and endothelial cells occur within 4 to 7 min, and both molecules are required to keep the sequestered neutrophils within the pulmonary capillary bed. Shedding of L-selectin occurs preferentially from the microvillar processes of neutrophils rather than the flat intervening regions. About 28% of L-selectin is still membrane-associated at 15 min of infusion and therefore available for interactions with endothelium. Shedding of L-selectin may occur more slowly *in vivo* than *in vitro*, perhaps because of antiproteolytic mechanisms prolonging L-selectin expression *in vivo* during the inflammatory response.

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